JC07 Rec'd PCT/PTO 04 JAN 2002

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	ATTORNEY'S DOCKET NUMBER SAE-005
	U S APPLICATION NO. (If known, see 37 C F R 1.5) 10/019783

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/04425	04 July 2000	05 July 1999

TITLE OF INVENTION:

THE MANUFACTURING OF IRON DEFICIENT RESISTANT GRASSES

APPLICANT(S) FOR DO/EO/US

Satoshi MORI et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendment to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendment has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern either document(s) or information included:

11. ☒ In Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16a. ☒ Notice to comply with sequence requirements with paper and disk.

16b. ☒ Petition under 37 CFR 1.84(a)(2) and (b)(2)

16c. ☒ Preliminary amendment with photographs.

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U.S. APPLICATION NO (If known, see 37 CFR 1.5) 10/019783	INTERNATIONAL APPLICATION NO PCT/JP00/04425	ATTORNEY'S DOCKET NUMBER SAE-005

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CRF 1.49(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....	**$ 890**	
International preliminary examination fee paid to USPTO (37 CRF 1.482)		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2).....		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 890**	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.49(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total 30 Claims	30-20=	10	X $18	**$ 180**	
Independent 1 Claims	1-3=	0	X $84	$	
Multiple dependent claim(s) (if applicable)			+ $270	**$ 270**	
TOTAL OF ABOVE CALCULATIONS			=	**$ 1340**	
Reduction by ½ for filing by small entity, if applicable.				$	
SUBTOTAL			=	**$1340**	
Processing fee of $130.00 for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.49(f)).			+	$	
TOTAL NATIONAL FEE			=	**$1340**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate sheet (37 CFR 3.28, 3.31). $40.00 per property			+	$	
TOTAL FEES ENCLOSED			=	$	
				Amount to be refunded.	$
				charged.	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 18-0013 in the amount of **$1340.00** cover the above fees. A duplicate of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **18-0013**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Rader, Fishman & Grauer, L.P.P.C.
1233 20th Street, N.W., Suite 501
Washington, DC 20036

Dated: January 4, 2002

SIGNATURE

David K. Benson
NAME

42,314
REGISTRATION NUMBER

10/019783
JC13 Rec'd PCT/PTO 0 4 JAN 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the U.S. Application of

Satoshi MORI et al.

Attn: Application Branch

Application No. To Be Assigned

Filed: Concurrently herewith

For: THE MANUFACTURING OF IRON DEFICIENT RESISTANT GRASSES

Commissioner of Patents and Trademarks
Box DAC
Washington, D.C. 20231

10019783 020402

PRELIMINARY AMENDMENT

Sir:

Prior to the initial examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Page 6, after line 21 (Brief Description of the Drawings), please insert the following paragraph:

-- The patent or application file contains at least one photograph executed in color. Copies of this patent or patent application publication with color photograph(s) will be provided by the Office upon request and payment of the necessary fee.--

IN THE CLAIMS:

Please cancel claim 4 without prejudice or disclaimer.

Please rewrite claims 1, 3, 5 and 7 as set forth below in clean form:

1. (amended) A method for producing gramineae comprising a step of introducing a genome gene that codes an enzyme in biosynthetic pathway of mugineic acids.

3. (amended) A method in accordance with claim 1 wherein a promoter used is

CaMV35S.

5. (amended) A method in accordance with claim 1 wherein the genome is a barley genome *naat*.

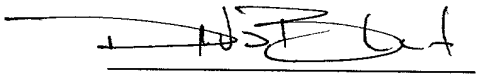
7. (amended) A gramineae with iron deficiency resistance manufactured through the method in accordance with any one of claims 1 to 3, 5 and 6.

REMARKS

This Preliminary Amendment is requested prior to the initial examination of the above-identified patent application to replace specification and to eliminate multiple dependency. Further, this Preliminary Amendment includes the Amendment under PCT Article 34. Additionally, in accordance with 37 CFR § 1.121(c))(1)(ii), amended claims 1, 3, 5 and 7 are set forth in a marked-up version in the pages attached hereto as APPENDIX.

If the Examiner has any suggestions for placing this application in even better form, the Examiner is invited to telephone the undersigned and the number listed below.

Respectfully submitted,



Date: January 4, 2002

David K. Benson
Reg. No. 42,314

RADER, FISHMAN & GRAUER, PLLC
The Lion Building
1233 20th Street, N.W., Suite 501
Washington, D.C. 20036
Tel: (202) 955-3750
Fax: (202) 955-3751
Customer No. 23353

APPENDIX

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims has been amended as follows:

1. (amended) A method for producing gramineae comprising a step of introducing a genome gene that codes an enzyme in biosynthetic pathway of mugineic acids.

3. (amended) A method in accordance with claim 1 [or 2] wherein [the] a promoter used is CaMV35S.

5. (amended) A method in accordance with claim [4] 1 wherein the genome is a barley genome *naat*.

7. (amended) A gramineae with iron deficiency resistance manufactured through the method in accordance with any one of claims 1 to 3, 5 and 6.

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25/pts

DESCRIPTION

THE MANUFACTURING OF IRON DEFICIENT RESISTANT GRASSES

Technical Field

The present invention relates to a manufacturing method for gramineae that have an iron deficiency resistance, gramineae obtained through the method, the method of growing said gramineae and the crop obtained through the method.

More specifically, the present invention relates to the creation of gramineae having an iron deficiency resistance by introducing genes, in which the gene codes an enzyme along the mugineic acid synthesizing route for gramineae, and more preferably, where said enzyme is nicotianamine amino transferase.

JP02/04502

Background Art

90% of the soil on the earth is inadequate soil that has some kind of problem. The inadequate soil, in general, lacks the elements essential to the growth of plants qualitatively and quantitatively, and therefore, the growth of plants is hindered or growth disorders occur due to the soil containing a large amount of heavy metals. Representative of inadequate soil is dryland salt accumulated soil. Of this type, there are ones in which NaCl and Na_2CO_3 are accumulated or $CaCO_3$ or $CaSO_4$ are accumulated in the topsoil due to artificial over-irrigation or dry weather over a long period of time. The halomorphic soil causes a salt density disorder, and the calcareous soil causes an iron deficiency disorder.

Approximately 30% of the cultivated soil on the earth is said to be a potentially iron deficient area. (Wallece et al. "Iron Chlorosis in Horticultual Plants," 75 American

Society for Horticultural Science, 819-839 (1960)). The calcareous soil in semiarid areas has calcareous components eluted from the core material due to a capillary effect and it is accumulated on the surface of the ground. In this soil, the pH is increased and becomes alkaline, and therefore, the iron in the soil exists in the form of Fe $(OH)_3$ and has extremely low solubility.

The plants grown in these soils have iron chlorosis and their growth is hindered or they die.

The iron obtaining system of higher plants is classified into two types, Strategy I and Strategy II. Strategy I is an iron obtaining system for higher plants excluding gramineae. It is a system in which the insoluble trivalent iron in the soil is reduced by the trivalent iron reduction enzyme that is present on the surface of the cell on the root, and then is it absorbed by the divalent iron transporter. Of those plants that have this system, there are ones that have a system to emit protons in the rhizosphere to increase the activity of the trivalent iron reduction enzyme by lowering the pH in the rhizosphere, and ones that have a system to emit phenol compounds in the rhizosphere and supply the Fe (III) to the trivalent reduction enzyme that is present on the surface of the cell by an Fe (III)-phenol compound chelate. Recent studies have isolated the transporter IRT1 (Eide et al, "A novel iron-regulated metal transporter from plants identified by functional expression in yeast," 93 Proc. Natl. Acad. Sci. 5624-5628 (May 1996)) that distinctively emerges on the root of the arabidopsis thaliana, and the gene for the trivalent reduction enzyme of the arabidopsis thaliana (Robinson et al., "The froh gene family from Arabidopsis thaliana: Putative iron-chelate reductases", 196 Plant and Soil 245-248, Kluwer Academic Publishers (1997)).

Strategy II is an iron obtaining system that is only observed in gramineae, which is one of the monocotyledons. The gramineae emits mugineic acids that have trivalent iron chelate activity under iron deficient conditions, and absorbs iron from the root as an "Fe (III)-mugineic acid" complex (Takagi et al. " Physiological aspect of magineic acid, a possible phytosiderophore of graminaceous plants," 7(1-5) Journal of Plant Nutrition 469-477 (1984)). There are 7 mugineic acids (MAs) that are known: mugineic acid (MA), 2'-deoxymugineic acid (DMA), 3-hydroxymugineic acid (HMA), 3-epihydroxymugineic acid (epiHMA), avenin acid (AVA), distichon acid and epihydroxydeoxymugineic acid (epiHDMA). All of the mugineic acids (MAs) are, as shown in FIG. 1, synthesized with methyonin as a precursor (Shojima et al. "Biosynthesis of Phytosiderophores", 93 Plant Physiol. 1497-1503 (1990) and Ma et al. "Biosyntheis of Phytosiderophores in several Triticeae species with different genomes," Vol. 50, No. 334, pp. 723-726, Journal of Experimental Botany, (1999)).

The excretion of mugineic acid has a circadian rhythm (Takagi et al. *supra*) and its excretion reaches a maximum after sunset, and there is no excretion during the night. In addition, it has been observed that the granule expands before the excretion in iron deficient barley and wilts after the excretion (Nishizawa et al., "The particular vesicle appearing in barley root cells and its relation to mugineic acid secretion," 10(9-16) Journal of Plant Nutrition 1013-1020 (1987)). Therefore, it is believed that the mugineic acid is synthesized in this granule. These fact indicates that the responding of the gramineae to the iron deficiency is formed by not only the synthesis of the mugineic acid but also is formed by a complicated system such as the transmission of an iron deficiency signal and changes in the root form.

It has been reported that a gene for the nicotianamine synthesizing enzyme, which is an enzyme related to the mugineic acid synthesizing route, has been isolated and it is induced by an iron deficiency. (Higuchi et al., "Cloning of Nicotianamine Synthase Gene, Novel Genes Involved in the Biosynthesis of Phytosiderophore," 119 Plant Physiology 471-479 (02/1999)). In addition, the gene for nicotinamine amino transferasegenes (NAAT) has been isolated and it is induced by an iron deficiency. (Takahashi et al., "Purification, characterization and DNA sequencing of nicotianamine aminotransferase (NAAT-III) expressed in Fe-deficient barley roots," Plant nutrition, 279-280, Kluwer Academic Publishers (1997))

Moreover, through differential screening using mRNA extracted from an iron deficient barley root and a control barley root, genes *Ids1, Ids2, and Ids3* which were specifically induced under iron deficient conditions have been isolated. The *Ids*1 is a gene that codes for metallothionain protein (Okumura et al., "An iron deficiency-specific cDNA from barley roots having two homologous cysteine-rich MT domains," 17 Plant Molecular Biology 531-533, Kluwer Academic Publishers (1991)). *Ids*2 is a gene in which the sequence of amino acids that is assumed from its genetic sequence is homologous to the hydroxide enzyme. (Okumura et al., "A dioxygenase gene (Ids2) expressed under iron deficiency conditions in the roots of Hordeum vulgare", Plant Molecular Biology 25; 705-719, Kluwer Academic Publishers (1994)) *Ids*3 is also a gene in which the sequence of amino acids that is assumed from its genetic sequence is homologous to the hydroxide enzyme. (Nakanishi et al., "Expression of A Gene Specifie for Iron Deficiency (Ids3) in the Roots of Hordeum Vulgare," 34(3) Plant Cell Physiol 401-410, JSPP (1993)) There are two hydroxide reactions along the epihydroxymugineic acid synthesizing route, and this

gene is believed to code the enzyme that catalyzes this reaction.

In addition, the examples of proteins that are induced by an iron deficiency of the barley root are, the IDS3 protein, adenin-ribose-phosphate transferases (Itai et al., "Induced activity of adenine phosphoribosyltransferase (APRT) in iron-deficient barley roots: a possible role for phytosiderophore production", Vol. 51, No. 348, pp. 1179-1188, Journal of Expeimental Botany (July 2000)), formicacid dehydrogenate enzyme (Suzuki et al. "Formate Dehydrogenase, an Enzyme of Anaerobic Metabolism, is induced by Iron Deficiency in Barley Roots," 116 Plant Physiol 725-732 (1998)), and 36kDa protein (Tomohiro Irifune, "Partial aminoacid sequences of a specific protein in iron-deficient barley root" (1991)). Gramineae biosynthesizes mugineic acid under iron deficient conditions. This time, it is believed that the methyonin contained in the root is reduced so that methyonin is synthesized during a methyonin cycle and at the same time, in order to convert the generated adnin into AMP, adenin-ribose-phosphate transferases are induced (Itai et al., *supra*).

The formic acid dehydrogenate enzyme decomposes formic acid generated during the methyonin cycle. It was reported that the root of a gramineae with an iron deficiency has a deformation of the mitochondrion and a reduction of the energy charge of the electron transmission system (Mori et al., "Why are young rice plants highly susceptible to iron deficiency", Iron nutrition and interactions in plants,175-188, Kluwer Academic Publishers (1991)). It is believed that the formic acid dehydrogenate enzyme is induced by the anaerobic condition generated by the iron deficiency, and that NADH is supplied as an energy source.

Along with the increase in population, an increase in food production is a

significant issue as a condition for human existence in the future. Gramineae has been one of the most important foods since ancient times, however, in reality, the growth of gramineae is difficult in areas with iron deficiencies. If it is possible to grow graminae in an area with an iron deficiency, an increase in food production would be possible, thus it has been attracting people's attention as one of the solutions to increase food production.

Disclosure of the Invention

The present invention has as an objective to provide gramineae with iron deficiency resistance, which can be grown in areas with iron deficiencies.

More specifically, the present invention has as an objective to provide graminae with an iron deficiency resistance that vigorously grows even in a calcareous alkaline soil by introducing the gene of an enzyme in the biosynthesis of the mugineic acid of gramineae to the gramineae.

The present invention relates to a manufacturing method for graminenae with improved iron absorbency by introducing a gene that codes an enzyme on the mugineic acid biosynthesis route to the gramineae. and more specifically, a gramineae with improved iron absorbency through the introduction of the gene *naat*, wherein said enzyme is nicotianamine amino transferase (NAAT).

Furthermore, the present invention pertains to a growing method of said gramineae with improved iron absorbency and crops obtained through said growth.

Brief Description of Drawings

FIG. 1 shows the mugineic acids' biosynthesis route for a barley root with an iron

deficiency and its rhizospheric environment.

FIG. 2 shows the genetic sequence of the binary vector pIG121Hm for a gramineae transformation in which the cDNA of naat-A is inserted.

FIG. 3 is a photo in place of a drawing that shows the results when detection of the introduced gene is carried out by the Southern Hybridization method. WT in FIG. 3 shows a case of an autochthon gramineae, and the control shows a control gramineae in which only the vector was introduced. 1-5, 1-6, 1-7, 8-1 and 15-2 show transformants having a 35S promoter.

FIG. 4 shows the result of measurement of NAAT activity in a root cultivated in a hydroponic solution in the presence of iron (+Fe) and an iron deficiency (-Fe). In FIG. 4, the whited out portion shows the case for +Fe and the shaded portion shows the case of -Fe. WT shows an autochthon type and 1-5, 1-6 and 1-7 show transformants.

FIG. 5 is a photo in place of a drawing that shows the growing state of each gramineae which is 8 weeks after a transplanting to alkaline soil. The control in FIG. 5 shows the control gramineae in which only the vector is transplanted and the gramineae on the right is the one that is transformed.

FIG. 6 is a graph that shows a transition of the height of each gramineae after being transplanted to alkaline soil. A black dot shows the transformer 15-2, a black square shows the transformer 8-1, and a white dot shows the control gramineae in which only the vector was transplanted.

FIG. 7 shows a limited enzyme map of a phage DNA including an isolated genome *naat*. In FIG. 7, E indicates *Eco*RI, H indicates *Hin*dIII, B indicates *Bam*HI, and N indicates *Not*I. The *Not*I site on both sides is the *Not*I located at the arm of λFIXII.

FIG. 8 shows the genetic sequence of the binary vector pBIGRZ1 for a transformed gramineae in which a fragment of the NAAT genome is inserted. In FIG. 8, NPTII is a kanamycin resistant gene, HPT is a hygromycin resistant gene, GUS is a β glucuronidase gene with intron, LacZ is a β galactosidase gene, 35P is a 35S promoter, NP is an NOS promoter, NT is an NOS terminator, MCS is a multi-cloning site, and Riori is an Ri plasmid replication starting point.

FIG. 9 is a drawing showing the base sequence of the obtained genome *naat*.

FIG. 10 is a drawing that shows the base sequence of *naat* and 5' upstream of *naat-A* and *naat*, the exon, the intron and 3' downstream, which were determined by comparing with the cDNA. In FIG. 10, the uppercase letters show the exon portion that is a transcription on the cDNA and the lowercase letters show the rest.

FIG. 11 is a schematic view of the obtained genome fragment. In FIG. 11, E is *Eco*RI, H is *Hin*dIII and B is *Bam*HI.

FIG. 12 shows the size of the intron in the cDNA of *naat-A* and *naat*.

FIG. 13 shows an amino acid expressed in a single letter code of an amino acid sequence of NAAT-A estimated from the cDNA.

FIG. 14 shows an amino acid expressed in a single letter code of an amino acid sequence of NAAT-B estimated from the cDNA.

FIGS. 15A and 15 B are photographs in place of drawings that show the growing state of each gramineae, ten (10) weeks after being transplanting to an alkaline soil. The control in FIGS. 15A and 15B shows the control gramineae in which only the vector is transplanted and the gramineae on the right is the one that is transformed with a genome *naat*.

FIG. 16 is a graph that shows the transition of the height of each gramineae in which a genome *naat* was introduced after being transplanted to alkaline soil. In FIG. 16, the gramineae on the left is the one transformed with *naat* and the one on the right is the control gramineae in which only the vector was transplanted.

Best Mode for Carrying Out the Invention

Said Strategy II, which is an iron obtaining system observed only in gramineae, from among the monocotyledon, utilizes a method of biosynthesizing and emitting mugineic acids to obtain iron. Therefore, the enhancement method of enzymes along the biosynthesis route of mugineic acids (see FIG. 1) was investigated.

The present inventors first paid attention to nicotianamine amino transferasegene (NAAT) as the enzyme on the mugineic acid synthesizing route, and then attempted to introduce the gene naat. Miraculously, it was found that gramineae with the gene introduced were able to vigorously grow even in a calcareous alkaline soil.

The cDNA of the naat-A, the nicotianamine amino transferasegenes (NAAT), was integrated into pIG121Hm using the *Xba*I and *Sac*I portion and the binary-vector shown in FIG. 2 was created. The obtained vector was used for transformation by introduction into an agro-bacterium.

The transformation of the gramineae was carried out in accordance with the method by Hiei et al., "Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA", 6(2) The Plant Journal 271-283, (1994), and "*Tsukinohikari*" was used as the material. The callus induced from the blastoderm was immersed and infected in said transformed

agro-bacterium suspension solution, and a regenerator (T1 plant) was obtained. Then, finally, the 34 strain transformed gramineae was obtained from seed.

The introduced gene was detected by the Southern Hybridization method. The results are shown in FIG. 3. In FIG. 3, WT shows a case of an autochthon gramineae and the control shows a case of a gramineae in which only the vector was introduced. 1-5, 1-6, 1-7, 8-1 and 15-2 show the transformer, which has a 35S promoter. As shown in FIG. 3, all the transformers have an over-generation of *naat-A*. In addition, it was found that among those 35S transformed gramineae, 8-1 and 15-2 had at least 5 copies and 2 copies of naat introduced, respectively.

By introducing the genetic naat, it is assumed that compared to the autochthon and one in which is it only introduced on the vector, there is an over-emission of nicotianamine amino transferase (NAAT) and as a result, the mugineic acid synthesizing route is activated, and consequently, the mugineic acids which are required for iron intake was massively produced.

Therefore, first, the NAAT activity of these species was investigated. Young plants (T2), 3 weeks after sprouting, were cultivated for 2 weeks in a hydroponic solution with the presence of iron (+Fe) and an iron deficiency (-Fe). The results of measurement of NAAT activity is shown in FIG. 4. In FIG. 4, the whited out portion shows the case of +Fe and the shaded portion shows the case of -Fe. WT shows the autochthon type and 1-5, 1-6 and 1-7 show the transformers.

As a result, for both +Fe and -Fe, the transformed ones had higher relative activity than the non-transformed autochthon one (WT), and in addition, it was found that the relative activity further increased with the -Fe condition. This shows that not only the

introduction of a gene allows the high activity of NAAT, but also, the transformer is significantly promoted with NAAT activity in the presence of an iron deficient state or conditions with an insoluble iron. In other words, it is assumed that it has become a species with a strong resistance to an iron deficient condition or the condition of insoluble iron.

From the above, it was found that the introduction of a gene *naat* promotes NAAT activity. Nonetheless, whether these transformers can be grown in actual iron deficient soil was investigated. When 35S-*naat-A* transformed gramineae was transplanted to an alkaline soil, its leaves turned yellow up to 2 weeks after the transplant, however, after 4 to 5 weeks, the new leaves became a dark green and started to recover. FIG. 5 is a photo showing the growth state 8 weeks after it was transplanted to alkaline soil. In FIG. 5, the control shows the control gramineae in which only the vector was transplanted and the gramineae on the right shows the transformed one. It is found in comparison to the control one, that the transformed one has significantly superior growth. In addition the transition of the plant height after it was transplanted to the alkaline soil is shown in FIG. 6. In the graph in FIG. 6, the Y axis shows the height of the plant (cm) and the X axis shows the number of days after it was transplanted to the alkaline soil. Black dots show transformer 15-2, black squares show transformer 8-1 and white dots show the control gramineae in which only the vector was transplanted.

As described above, 35S transformed gramineae 8-1 has at least 5 copies of *naat* genes, and 35S transformed gramineae 15-2 has at least 2 copies of *naat* genes introduced. From the height of the plant in FIG. 6, the number of copies of the gene is not related and it shows that as long as the gene was introduced, the gramineae has gained an iron deficiency resistance.

As described above, the introduction of the gene increases the activity of the enzyme along the mugineic acid synthesizing route of the gramineae, and furthermore, it was found that by doing so, it added an iron deficiency resistance.

For the enzyme along the mugineic acid synthesizing route of the present invention, it is acceptable as long as it is an enzyme along the mugineic acid biosynthesizing route shown in FIG. 1, and the introduction of the gene that codes said enzyme increases its activity. As described above, among the candidates, nicotianamine amino transferase (NAAT) and nicotinamine synthesizing enzyme are desirable. The gene that codes the enzyme along the mugineic acid synthesizing route of the gramineae of the present invention can be either cDNA or one derived from the genome. As described in the later section, the use of a genome is a preferable example of the present invention.

Therefore, for the gramineae of the present invention, it is acceptable as long as it is a plant that can absorb iron through the Strategy II system, and it is not limited to gramineae in the academic sense. The preferable examples of gramineae of the present invention are, rice plants, corn, sorghum, wheat, barley, and oats. These gramineae can have the method of the present invention applied regardless of its species, and the gramineae with the target gene introduced can be manufactured.

The promoter of the present invention is not limited as long as it can generate the target enzyme. 35S promoter, and more specifically, CaMV35S promoter can be used.

The vector of the invention is not specifically limited as long as it can preferably be used during the transformation. The transformation method of the present invention is not limited to said method that uses the agro-bacterium method and a variety of transforming methods using particle guns, etc., can be employed. The cells of the

gramineae formed are not limited to the cell from said callus and a variety of cells can be used, however, normally it is preferable to use ones derived from the callus.

The iron deficiency related to the present invention can be a state where iron is deficient, but preferably it is a state where the form of the iron is one that the plant has problems absorbing, and depending on the type of plant, it can be determined whether it is deficient of iron or not. Therefore, the definition of the iron deficiency resistance in the present invention is that the resistance is to the difficult conditions where the plants of the subject have difficulty absorbing the iron in the soil.

Next the present inventor attempted an introduction of a genome *naat* instead of the cDNA of the *naat*.

For the genome *naat*, the library (manufactured by Srtratagen Corp.) created using the genome DNA extracted from barley (*Horudeum vulgare L. var. Igri*) was used. This library was partially cut by limiting the enzyme Sau3AI and was introduced to the *Xho*I site of the λFIXII vector. For the probe, the entire cDNA of the *naat-A*, which was isolated in advance was used. An *Escherichia coli* XL1-Blue (*E. coli XL1-Blue MRA (P2)*) was used as the host.

As the result of screening, five (5) phages were obtained. From these, each of the phage DNA was isolated and a limiting enzyme map was created. It was found that the same fragments were increased for all the cases. Namely, it was found that the obtained five phages were derived from the same portion of the genome. It was assumed that it contains the *naat* used in the probe. Therefore, the base sequence determination for the one of them shown in FIG. 7 was carried out.

The phage DNA shown in FIG. 7 is inserted in the *Not*I site of the plasmid

vector-pBIGRZ1 in which 10 kb or more fragments can be inserted and a transformation of the gramineae using *agro-bacterium* can be carried out. (See FIG. 8.)

In addition, up to 11.0 kg of the fragments shown in FIG. 7 were divide into 4 parts, that is A to D, and they were introduced to the *Eco*RI site (B, C) of the plasmid vector-pBluescript SK(i) or the *Not*I and the *Eco*RI site (A, D).

For fragments A to D, the base sequence was determined from both sides of the fragment using a primer based on the sequence on the plasmid (M13 forward primer, M13 reverse primer). To determine the DNA base sequence, the DNA sequencer DSQ-2000L of Shimadzu, Ltd. was used.

The details of the sequence determination are shown in the embodiments.

Sequence No. 1 in the sequence table shows the determined 10.966 bp base sequence. In addition, the entire sequence is shown in FIG. 9 (without base number).

From the obtained base sequence, this 10,966 bp gene is found to be a fragment of the barley genome that codes the *naat-A* and *naat* that have been obtained. It was in the order of *naat* and *naat-A*.

5' upstream *of naat-A* and *naat*, the intron and 3' downstream were determined by comparing with the 10th cDNA as shown. In FIG. 10, the upper case letters show the exon portion transcribed to the cDNA, and the lower case letters show the rest. The base number of the exon portions are as follows.

FIG. 11 shows the schematic view thereof. The exon portion is shown in the shaded portion. Both genes comprise 6 intron and 7 exon. In addition, the insertion location of the intron was homologous for each of the genes. FIG. 12 shows the location in the cDNA and what size of intron was inserted.

The amino acid sequence of *naat-A* and *naat* estimated from the cDNA, is shown in FIGs. 13 and 14, respectively.

The transformation method of gramineae, in which an obtained barley genome *naat* is introduced, was carried out in accordance with the transformation method of said 35S transformed gramineae.

Next, the inspection of the iron deficiency resistance of the gramineae introduced with the obtained genome *naat* was carried out. Of the obtained regenerators (T 1), 39 individuals and 15 individual controls, in which only the vector was introduced, were used, and the inspection was carried out in a similar manner to the 35S transformed plant. From the 5th week after the transplantation, the height of the plants was measured every week or every other week. Twice every 4 to 5 weeks, they were transplanted to a pot with increased soil.

The leaves of the transformed gramineae with the genome *naat* turned yellow by the second week after they were transplanted to the alkaline soil, however, during the 4th to 5th week, new leaves started to become dark green and recover, and then they started to show vigorous growth. Compared to this, the control group in which only the vector was introduced continued to have yellow leaves for a long period of time, and from around the 8th week, new leaves started to turn green.

FIGS. 15A and 15B are photographs that show the growth state after it was transplanted to the alkaline soil. The control in FIGS. 15A and 15B show the control gramineae in which only the vector was transplanted. The gramineae on the right is the one transformed with the genome *naat*.

FIG. 16 shows the transition of the plant height after it was transplanted to the

alkaline soil. In the graph of FIG. 16, the Y axis shows the plant height (cm), and the X axis shows the number of days after it was tranplanted to the alkaline soil. In FIG. 16, the gramineae on the left shows the gramineae transformed with a genome *naat* and the one on the right shows a control gramineae in which only the vector was transplanted.

From the above, it was found that the introduction of the genome *naat* allows the gramineae to have additional iron deficiency resistance.

Examples

The present invention is described in detail using embodiments as follows, however, the present invention is not limited to these.

Example 1: Transforming Method of Gramineae in Which There is an Over-developing *Naat* with CaMV35S Promoter

The binary vector shown in FIG. 2 was created by integrating the cDNA of the genetic *naat* to pIG121Hm, using the *Xba*I and *Sac*I portions. These were introduced to agro-bacterium and used for the transformation.

The transformation of gramineae was carried out in accordance with the method of Hiei, et al., "Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA", 6(2) The Plant Journal 271-283, (1994) and "*Tsukinohikari*" was used as the material. First, hulled seeds were sterilized and seeds on a callus-inducing medium (pH 5.8) comprising N6 inorganic salt, 30 g/L of N6 vitamin, 2 mg/L of sucrose, 2, 4-D, and 2 g/L gelrite were cultured for 3 weeks at 25 °C under the conditions of 60 μmol/m2s with a 16-hour photo period/8 hour

dark period, and the callus was induced from the blastoderm.

After it was transplanted to the new medium and cultured for 3 days at 25 °C in a bright place, it was immersed in the agro-bacterium suspension with an agro-bacterium suspension medium (pH5.8) (20 g/L of AA inorganic salt, amino acid, B5 vitamin, 2 mg/L of sucrose, 0.2 mg/L of 2, 4-D, 10 mg/L of kinetin and acetosyringone). Then after it was dried with a paper towl, it was infected for 3 days at 28 °C in a dark place in the co-existing culturing medium (pH5.2) (30 g /L of N6 inorganic salt and N6 vitamin, 10 g/L of sucrose, 2 mg/L of glucose, 10 mg/L of 2, 4-D, and 2 g/L of acetosyringone gelrite).

Then, the agro-bacterium was removed by rinsing the callus with a sterilized washing solution with 500 mg/L of Claforan, and it was placed on a selected medium containing 50 mg/L of hygromycin (pH5.8) (30 g/L of N6 inorganic salt and N6 vitamin, 2 mg/L of sucrose, 2 g/L of 2, 4-D, 500 mg/L of gelrite, 50 mg/L of Claforan and hygromycin) and cultured for 3 weeks at 25 °C in a bright place.

After it was cultured, it was transferred to a redifferentiation medium (pH5.8) (30 g/L of MS inorganic salt and MS vitamin, 30 g/L of sucrose, 2g/L of sorbitol, 1 mg/L of casamino acids, 2 mg/L of NAA, 500 mg/L of BAP, 50 mg/L of Claforan, 4 g/L of hygromycin and gelrite) and the regenerator (T1 plant) that was redifferentiated in 3 to 5 weeks was transferred to an inspection medium. The inspection medium (pH5.8) comprises 30 g/L of MS inorganic salt and MS vitamin, 50 mg/L of sucrose, 8 g/L of hygromycin, and agar.

The plants that grew to fill the petridish were established for 4 to 5 days, transferred to a soil mixed with a synthetic culture soil (BONSOL 1, Sumitomo Chemical Co., Ltd.) and Vermiculite in a 1:1 ratio and seeds were obtained. Consequently, a 34 strain

transformed gramineae was obtained.

Example 2: Detection of the Introduced Gene Using the Southern Hybridization Method

The leaves of the T1 plant obtained in Embodiment 1 were ground and the genome was extracted by a modification of the C-TAB method. The extracted genome was treated with *Hin*d III and separated by electrophoresis using a 0.8% agarose gel. These were blotted on a nylon membrane. Using the primer created with the internal sequence of the *naat* on a probe, and one labeled with 32 P by PCR, hybridization was carried out. Then the band was detected using BAS 2000 (Fuji Photo Film Co., Ltd.).

The results of this Southern Hybridization are shown in FIG. 3. In FIG. 3, WT shows the autochthon gramineae, and the control shows a control gramineae in which only the vector was introduced. 1-5, 1-6, 1-7, 8-1 and 15-2 are cases in which the gramineae has an over-emergence of *naat-A* with the 35S promoter.

From the results shown in FIG. 3, it was found that of the 35S transformed gramineae, at least 5 copies and 2 copies of *naat* were introduced to the 8-1 and 15-2, respectively.

Example 3: Inspection of Iron Deficiency Resistance Using Alkaline Soil

As a sample soil, a fossil shell soil comprised of the following composition was used. The content of each element is shown as % dray soil after analysis by several methods, dry weight method after ashing, flame photometry, atomic absorption, spectrophotometry and etc. Some elements were shown as chemical compounds, Therefore, summing up all data covers more than 100% because some elements were

doubly counted.

Water content	0.48%
Total phosphate	0.12%
Total potassium	0.12%
Total silicate	22.79%
Total lime	37.82%
Total magnesia	0.91%
Total manganese	0.018%
Total boron	0.003%
Alkaline	38.80%
Hydrochloride insoluble substance	28.88%
Iron oxide	0.99%
Aluminum oxide	5.59%
Zinc	0.002%

EP 0 529 854 B1

The soluble iron content of this soil was 22 ppm, the pH was 8.78 and the electric resistance was 0.03 mΩ.

The inspection of the 35S transformed plant was carried out such that, first, the obtained seeds from the regenerator (T1) were sown on an MS solid medium containing 50 mg/L and selected, and after they were established, young plants (T2) that grew to 20 to 25 cm were used.

The inspection of the resistance was carried out for 16 of the 34 strains having 27 species. The inspection method is as follows: first, a paper towel and a filter were cut in a circle and placed on the bottom of a plastic black pot (0.5 L) and filled with alkaline soil. Plants were transferred to the pot and then from the bottom of the pot placed in a hydroponic solution (Kasugai Solution: 7×10^{-4}M K_2PO_4, 1×10^{-4}M KCl, 1×10^{-4}M

KH_2PO_4, 2×10^{-3}M $Ca(NO_3)_2$, 5×10^{-4}M $MgSO_4$, 1×10^{-5}M H_3BO_3, 5×10^{-7}M $MnSO_4$, 5 $\times 10^{-7}$M $ZnSO_4$, 2×10^{-4}M $CuSO_4$, 1×10^{-8}M $(NH_4)_3MoO_{24}$, 1.5×10^{-4}M Fe-EDTA) at 2 to 3 cm from the bottom of the pot, and grown in a greenhouse at a temperature of 30 °C during the day and 25 °C at night. The alkaline soil was increased to 1L after 3 to 4 weeks and after 8 to 9 weeks, increased to 2L and transferred. From the second week after the transplantation, the plant height was measured every week or every other week.

The measurement of the NAAT relative activity of the transformed gramineae (35S-naat gramineae) grown in the hydroponic solution of +Fe (iron presence) or -Fe (iron deficiency) was carried out as follows. Young plants (T2), 3 weeks after the sprouting, were grown with an +Fe and -Fe hydroponic solution, and the NAAT activity at the root of each plant was measured. The results are shown in FIG. 4. In FIG. 4, the whited out portion shows the case for +Fe, and shaded portion shows the case for -Fe. WT shows the autochthon case and 1-5, 1-6 and 1-7 show the transformed cases.

In both the +Fe and –Fe case, the transformed one had a higher relative activity than the non-transformed autochthon (WT) and the relative activity was even higher in the case of -Fe. (See FIG. 4)

Example 4: Inspection of iron Deficiency Resistance Using Alkaline Soil

35S-*naat-A* transformed gramineae were transferred to an alkaline soil and their growth was observed.

The leaves of the 35S-*naat-A* transformed gramineae turned yellow until the second week after the transplant, however, on the 4th to 5th week, the new leaves turned to a deep green and recovered. Thus it was found that the introduction of *naat* allows the

gramineae to have an iron deficiency resistance.

FIG. 5 is a photo showing the growth state at 8 weeks after the transplant to the soil. In FIG. 5, the control shows the control gramineae in which only the vector was transplanted. The gramineae on the right is transformed.

FIG. 6 shows the transition of the plant height after being transplanted to the alkaline soil. In the graph of FIG. 6, the Y axis shows the plant height (cm) and the X axis shows the number of days after it was transplanted to the alkaline soil. Black dots show transformer 15-2, black squares show transformer 8-1 and white dots show the control gramineae in which only the vector was transplanted.

Ir was found that by introducing a gene naat, an iron deficiency resistance could be added to the gramineae. (See FIGs. 5 and 6.)

Example 5: Isolation of *Naat-A* and *B* Genomic Clone

The screening procedure was carried out in accordance with Itaru Watanabe, "*Cloning and Sequence,*" Nosonbunka (1989).

The λ FIXII library purchased from Stratagnen Corp. was used as the library. This is created using genome DNA extracted from barley (*Horudeum vulgare L. var. Igri*). The genome DNA was partially cut by the limiting enzyme, and introduced to the *Xho*I site of the λ FIXII vector. The insertion size of the library was 9 to 23 kb.

(1) *E. coli* (XL1-BLUE MRA (P2)) was cultured overnight in a NZCYM liquid medium, (10 g of NZ amine, 5 g of NaCl, 1 g of casamino acid, 5 g of Bacto-yeast extract, 2 g of $MgSO_4$ $7H_2O$, and approximately 6 mL of 1N NaOH was diluted with 1 L of distilled water (pH 7.5) and sterilized with an autoclave) then centrifuged and then it was

suspended in 20 mL of 10mM $MgSO_4$ solution.

(2) 100 mL of this E-coli suspension solution and 100 mL of phage dilution (the amount in which 25,000 plaque are created on the plate for screening (9cm x 13 cm)) were mixed and left for 20 minutes at 37 °C, then it was mixed with 8 mL of 50 °C 0.7% top agar (0.7 g of agarose was added per 100 mL) and sown on the plate for screening (9 cm x 13 cm). The plate was left at 37 °C and then it was cultured until the size of the plaque reached 0.5 mm.

(3) A nylon membrane, HYBOND-N (Amersham Corp.) was cut to the size of the plate and then placed on top of topagarose for 30 seconds. This was placed on a filter dipped with a denaturation solution (0.5 M NaOH, 1.5 M NaCl) with the side that came in contact with the plaque up. A second membrane was placed on the topagarose, and left for one minute. Similarly, it was placed on the filter dipped in the denaturation solution. After it was left for 5 minutes, the second membrane was moved onto a filter dipped with a neutralization solution (0.5M Tris-HCl, pH 8.0, 1.5M NaCl). After it was left for 5 minutes, it was well washed twice with 2xSSPE (0.02M, NaH_2PO_4 pH 7.4, 0.3M NaCl, 2 mM EDTA) and then dried.

(4) In order to use the whole length of the cDNA of the isolated *naat-A* in advance, those that were at the site of the *Hin*dIII of the plasmid vector pYH23 and the *Not*I sites were cut out and purified. These were labeled with [α^{-32}P] dATP using a RANDOM PRIMER DNA LABELLING KIT VER. 2 (Takara Shuzo Co., Ltd.)).

Prehybridizaqtion was carried out for 1 hour at 65 °C with 30 mL of hybridization buffer (6 x SSPE、 5 x Denhart solution, 0.1% SDS, 100 mg/ mL altered salmon spermary DNA) that was preheated to 65 °C, and the hybridization buffer was replaced (25 mL).

The probe prepared as described above was added to this, and hybridization was carried out for 12 hours at 65 °C. The membrane was cleaned with a cleaner (5x SSPE) heated to 65 °C in advance twice for 10 minutes, and once with a highly stringent cleaner (2 x SSPE, 0.1% SDS) at 65 °C. The membrane was wrapped with Saran Wrap, and photosensitized overnight to an imaging plate (Fuji Photo Film Co., Ltd.) and results were obtained with an imaging analyzer (Fuji Photo Film Co., Ltd.).

The reagent used is such that 20 x SSPE (0.2 M NaH_2PO_4 pH 7.4, 3 M NaCl and 20 mM EDTA), 50 x denhart solution, 5g of FICOLL 400, 5g of polyvinylpyrrolidone (MW 360,000) and 5g of calf serum albumin were dissolved in 500 mL of distilled water and filtered with a 0.45 mm filter.

(5) What emerged on both of the two membranes was determined to be positive and the plaque that corresponded to the location was cut out from the petridish. That which was cut out was placed in an SM solution (50 mM Tris-HCl pH7.5, 0.1M NaCl, 7mM MgSO4, and 0.01% gelatin) and stored at 4 °C. Then, using this phage solution, a second and third screening was carried out in a similar manner. In the end 5 phages were obtained.

The phage DNA of each of the five phages obtained as described above was isolated and a limiting enzyme map was created. It was found that the same fragments were increased for all the cases. Namely it was found that all of the obtained 5 phages were derived from the same part of the genome. In addition, it was assumed that the *naat* used for the probe was contained. Therefore the base sequence was determined for one of these. (See FIG. 7.) In FIG. 7, E is *Eco*RI, H is *Hin*dIII, B is *Bam*HI and N is *Not*I. The *Not*I site at both sites is the *Not*I on the arm of λFIXII.

Example 6: Sub-cloning and Determination of the Base Sequence

(1) The phage DNA shown in FIG. 7 is inserted in the NotI site of the plasmid vector-pBIGRZ1 in which the 10 kb or more fragments can be inserted and a transformation of the gramineae using agro-bacterium can be carried out. (See FIG. 8.) 11.0 kbp of fragments, from the first *Not*I site to the *Not*I site located at 11.0 kb of the phage DNA shown in FIG. 7 was cut out and inserted at the *Not*I site of the pBIGRZ1. (See FIG. 8.) In FIG. 8, NPTII is a kanamycin resistant gene, HPT is a hygromycin resistant gene, GUS is a β glucuronidase gene with intron, LacZ is a β galactosidase gene, 35P is a 35S promoter, NP is an NOS promoter, NT is an NOS terminator, MCS is a multi-cloning site, and Riori is an Ri plasmid replication starting point.

In other words, the base sequence was determined for this 11.0 kb. For the transforming of the rest of the gramineae, this created construct was used.

(2) pBIGRZ1 is stably maintained in the *E. coli* (XL1-BLUE). *E-coli* having this construct were cultured and the plasmid was extracted from here using plasmid adjuster PI-50 α. (Kurashiki Boseki Co., Ltd.)

(3) The fragments up to 11.0 kb that are shown in FIG. 7 are classified into 4 sections of A to D and these were introduced to the *Eco*RI site (B. C) of the plasmid vector of pBluescript SK (-) or the *Not*I and *Eco*RI site (A, D).

(4) The base sequence from both sides of the fragments for the fragments A to D was determined by the primer (M13 forward primer, M13 reverse primer) based on the sequence on the plasmid. The DNA sequencer DSQ-2000L from Shimadzu, Ltd. was used to determine the base sequence.

(5) The primer to read further from the portion of the base sequence was

determined for each fragment and created, and the primer to confirm the already-read sequence in reverse was created and the base sequences were determined in series. At the end, the sequence was determined for all the fragments in both directions from 5' and 3'. The sequence of the primers used to determine the base sequence of each fragment is shown as follows.

These primers are labeled with the fluorescein isothiocyanate, FITC, at the 5' edge in order to be used by the DSQ-2000L.

Primers for fragment A

Name: Sequence F-A1F: FITC-5'-gct act agt agt att cct ggt gta g

Name: Sequence F-A1R: FITC-5'-gga gta cta cta gac tac acc agg a

Name: Sequence F-A2F: FITC-5'-aca tgc gca tgc atg aat tgc cg

Name: Sequence F-A2R: FITC-5'-caa ttc atg cat gcg cat gtg cc

Primers for fragment B

Name: Sequence F-B1F: FITC-5'-ggt caa gta tgc agt atg ttg gaa c

Name: Sequence F-B1R: FITC-5'-gtt cca aca tac tgc ata ctt gac c

Name: Sequence F-B2F: FITC-5'-cta gaa gcc tat gga tgt ttc ttt tgg

Name: Sequence F-B2R: FITC-5'-cca aaa gaa aca tcc ata ggc ttc tag

Name: Sequence F-B3F: FITC-5'-agt tct tat caa ttt ccg aga tga c

Name: Sequence F-B3R: FITC-5'-ata gtc atc tcg gaa att gat aag a

Name: Sequence F-B4F: FITC-5'-agt ggt cac cat gcg gac caa cac c

Name: Sequence F-B4R: FITC-5'-ggt gtt ggt ccg cat ggt gac cac t

Primers for fragment C

Name: Sequence F-C1F: FITC-5'-cac cgg cca gtt caa ctg cta cgc

Name: Sequence F-C1R: FITC-5'-gcg tag cag ttg aac tgg ccg gtg

Name: Sequence F-C2F: FITC-5'-ttt gga gga gat cca tga cga cat a

Name: Sequence F-C2R: FITC-5'-tat gtc gtc atg gat ctc ctc caa a

Name: Sequence F-C3F: FITC-5'-tct tct cat atg cta ctg tgg gga t

Name: Sequence F-C3R: FITC-5'-tga cat gca aca cag gga cat gag c

Primers for fragment D

Name: Sequence F-D1F: FITC-5'-cat gct gac gaa gag cga ggt cat a

Name: Sequence F-D1R: FITC-5'-ccc agg ata tga cct tag tgg ttg g

(6) For the portion of the sequence that could not be determined completely, the following primers were newly synthesized by using the ABI PRISMTM 310 genetic analyzer that is an automatic DNA sequencer from PerkinElmer Japan, Inc.

Primers for fragment B

Name: Sequence B5F: 5'-gaa tgg caa act ggg tcc gca tta c

Name: Sequence B5R: 5'-gta atg cgg acc cag ttt gcc att c

Name: Sequence B6F: 5'-ctg gtt gtt gtg gcc tgg acg aaa c

Name: Sequence B6R: 5'-gtt tcg tcc agg cca caa caa cca g

Name: Sequence B7F: 5'-agc aca aac cct acc tat gtt agg c

Name: Sequence B7R: 5'-gcc taa cat agg tag ggt ttg tgc t

Primers for fragment C

Name: Sequence C4F: 5'-tgg aat ttc gcc cgg ggc aag gac

Name: Sequence C4R: 5'-ccc tgt gac aag tgc tct gct acg

Name: Sequence C5F: 5'-tct ggg atc tca gtg cat cca aca

Name: Sequence C5R: 5'-gaa gca tat atc agt caa aca taa cc

In addition, in order to determine the junction of the fragments A and B and fragments B and C, the following primers were created. The base sequence was determined for the construct created in (1) using the ParkinElmer Japan, Inc. automatic DNA sequencer ABI PRISMTM 310 genetic analyzer.

Border between fragments A and B

Name: Sequence A-eF: 5'-cac atc ctt tgc ctt gct gaa tat gg

Name: Sequence B-tR: 5'-cag tag tac taa tta atc acc tta gta gc

Border between fragments B and C

Name: Sequence B-eF: 5'-cac gat caa cca aag aat gtc ctc c

Name: Sequence C-tR: 5'-tac ttg tat atg cag ctc cag cac

(7) The sequence number 1 in the sequence list of the determined 10,966 bp base sequence is shown. The entire sequence is shown in FIG. 9 (without base numbers).

From the obtained base sequence, it was found that this 10,966 bp gene is a fragment of the barley genome that codes the *naat-A* and *naat-B* obtained so far. The order was *naat-B* and *naat-A*.

At the 109th location, 5' upstream, exon, intron, and 3' downstream of naat-A and naat-B were determined by comparing with the cDNA as shown. In the 10th location, uppercase letters represent the exon portion transplanted to cDNA and lowercase letters represent the rest. The base numbers for the exon portion are as follows.

naat-B

First exon 579-1299 (Starting codon 6518)

Second exon 1483-1825

Third exon 1922-2140

Fourth exon 2244-2303

Fifth exon 2761-2916

Sixth exon 3263-3356

Seventh exon 3735-4033 (Ending codon 3868)

naat-A

First exon 6457-6897 (Starting codon 6518)

Second exon 7029-7371

Third exon 7479-7697

Fourth 4 exon 7784-7843

Fifth exon 8285-8440

Sixth exon 8738-8831

Seventh exon 9414-9732 (Ending codon 9547)

This schematic view is shown in FIG. 11. The exon portion is shown as the shaded portion. Both genes are formed with 6 introns and 7 exons. In addition, the location of the insertion of the intron was homological. FIG. 12 shows where in the cDNA, and which size of intron was inserted. FIGs. 13 and 14 show the amino acid sequence of *naat-A* and *naat-B* estimated from the cDNA.

Example 7: Transforming Method of Gramineae Introduced with a Barley Genome *Naat*

The transformation method of the gramineae introduced with the genome naat of barley obtained in Example 6 described above was carried out in a similar manner to the

35S transformed gramineae except at those points shown in (1) to (3) as follows.

(1) The callus induction was carried out at 28 °C in a dark place, and the callus induction medium comprised 0.3 g/L of N6 inorganic salt and N6 vitamin, 30 g/L of casamino acid, 2 mg/L of sucrose, 2.8 g/L of 2, 4-D, 4 g/L of proline and gelrite (pH5.8).

(2) It was infected with agro-bacterium at 25 °C and a coexisting culture medium comprising 30 g/L of N6 inorganic salt and N6 vitamin, 10 g/of sucrose, 1 g/L of glucose, 2 mg/L of casamino acid, 20 mg/L of 2, 4-D, 2 g/L of acetosyringone and gelrite (pH5.2). It was carried out with a placement of the filter.

(3) For the selection, a selection medium was used containing 10 mg/L for the first week, 30 μg/L for the next week and 50 mg/L for the last two weeks of hygromycin, and it was cultured at 28 °C in a dark place.

The selection medium comprising 1 g/L of N6 inorganic salt and N6 vitamin, casamino acid, 30 g/L of sucrose, 2 mg/L of 2, 4-D, 250 mg/L of Claforan, 10 to 50 mg/L of hygromycin and 2 g/L of gelrite (pH5.8) was used and a redifferentiation medium comprising 30 g/L of MS inorganic salt and MS vitamin, sucrose, 30 g/L of sorbitol, 2 g/L of casamino acid, 1.1 g/L of MES, 2 mg/L of NAA, 1 mg/L of kinetin, 250 mg/L of CLAFORAN (Hoechst Marion Roussel Ltd. Japan), 50 mg/L of hygromycin, and 4 g/L of gelrite (pH5.8) was used and it was cultured at 28 °C.

Example 8: The Inspection of the Iron Deficiency Resistance of Gramineae with a Genome *Naat* Introduced

The inspection of the iron deficiency resistance of gramineae with a genome *naat* introduced was carried out for 39 individuals out of the obtained regenerators (T1) and 15

controls in which only the vector was introduced in a similar manner to the 35S transformed plant. From the 5th week after the transplant, the height of the plants was measured every week or every other week. They were transferred twice every 4 to 5 weeks to a pot with an increased soil size.

The leaves of the transformed gramineae with a genome *naat* turned yellow by the second week after they were transplanted to an alkaline soil, however, on the 4th to 5th week, the new leaves started to become a dark green and recover, and then started to show vigorous growth. Compared to this, the control group in which only the vector was introduced continued to have yellow leaves for a long period of time, and from approximately the 8th week, new leaves started to turn green. From this fact, it was found that the introduction of *naat* allows the gramineae to have an iron deficiency resistance. (See FIGs. 15 and 16.)

FIGS. 15A and 15B are photographs that show the growth state after being transplanted to an alkaline soil.

FIGS. 15A and 15B show the control gramineae in which only the vector was transplanted. The gramineae on the right was transformed with a genome *naat*.

The transition of the height of the plants after being transplanted to an alkaline soil is shown in FIG. 16. In FIG. 16, the Y axis shows the height of the plant (cm) and the X axis shows the number of days after it was transplanted to the alkaline soil. In FIG. 16, the one on the left is the gramineae transformed with a genome *naat* and the one on the right is the control gramineae in which only the vector was transplanted.

Through this, it was found that the introduction of a genome *naat* allows an increase in the iron deficiency resistance for a gramineae.

Industrial Applicability

The present invention provides a new gramineae with iron deficiency resistance and provides a new gramineae that can be grown in a cultivation area with an iron deficiency.

In addition, the present invention provides the new knowledge that a gramineae with improved iron absorbency can be obtained by introducing a gene that codes the enzyme along the mugineic acid biosynthesizing route, to a gramineae.

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CLAIMS

1. A method for producing gramineae comprising a step of introducing a gene that codes an enzyme in biosynthetic pathway of mugineic acids.

2. A method in accordance with claim 1 wherein the enzyme is nicotianamine amino transferase (NAAT) and the gene that codes thereof is *naat*.

3. A method in accordance with claim 1 or 2 wherein the promoter is CaMV35S.

4. A method in accordance with any one of claims 1 to 3, wherein the gene is a genome gene.

5. A method in accordance with claim 4 wherein the genome is a barley genome *naat*.

6. A method in accordance with claim 5 wherein the base sequence of the gene is the base sequence shown in sequence No. 1 in the sequence list, or a base sequence that can be hybridized under stringent conditions for said base sequence as well as having it possible to generate a protein having nicotianamine amino transferase (NAAT) activity, in addition to the base sequence complementary to thereof.

7. A gramineae with iron deficiency resistance manufactured through the method in accordance with any one of claims 1 to 6.

8. The seeds of gramineae in accordance with claim 7.

9. The cells of gramineae in accordance with claim 7.

10. A method of growing gramineae in an iron deficient field in accordance with claim 7.

11. A crop of gramineae obtained through the method in accordance with claim 10.

ABSTRACT

A gramineous plant having tolerance to iron deficiency which can grow even in an area with ion deficiency. More particularly, a gramineous plant having tolerance to iron deficiency and capable of vigorously growing even in calcareous alkaline soil which is constructed by transferring a gene of an enzyme in the pathway of the biosynthesis of mugineic acids in gramineous plants into a gramineous plant. A method of constructing a gramineous plant having improved iron absorbability which comprises transferring a gene encoding an enzyme (preferably nicotianamine aminotransferase; NAAT) in the pathway of the biosynthesis of mugineic acids into a gramineous plant; a gramineous plant constructed by the above method; a method of cultivating the above gramineous plant having improved iron absorbability; and a crop obtained by the cultivation. Namely, the constructing of a novel gramineous plant having tolerance to iron deficiency.

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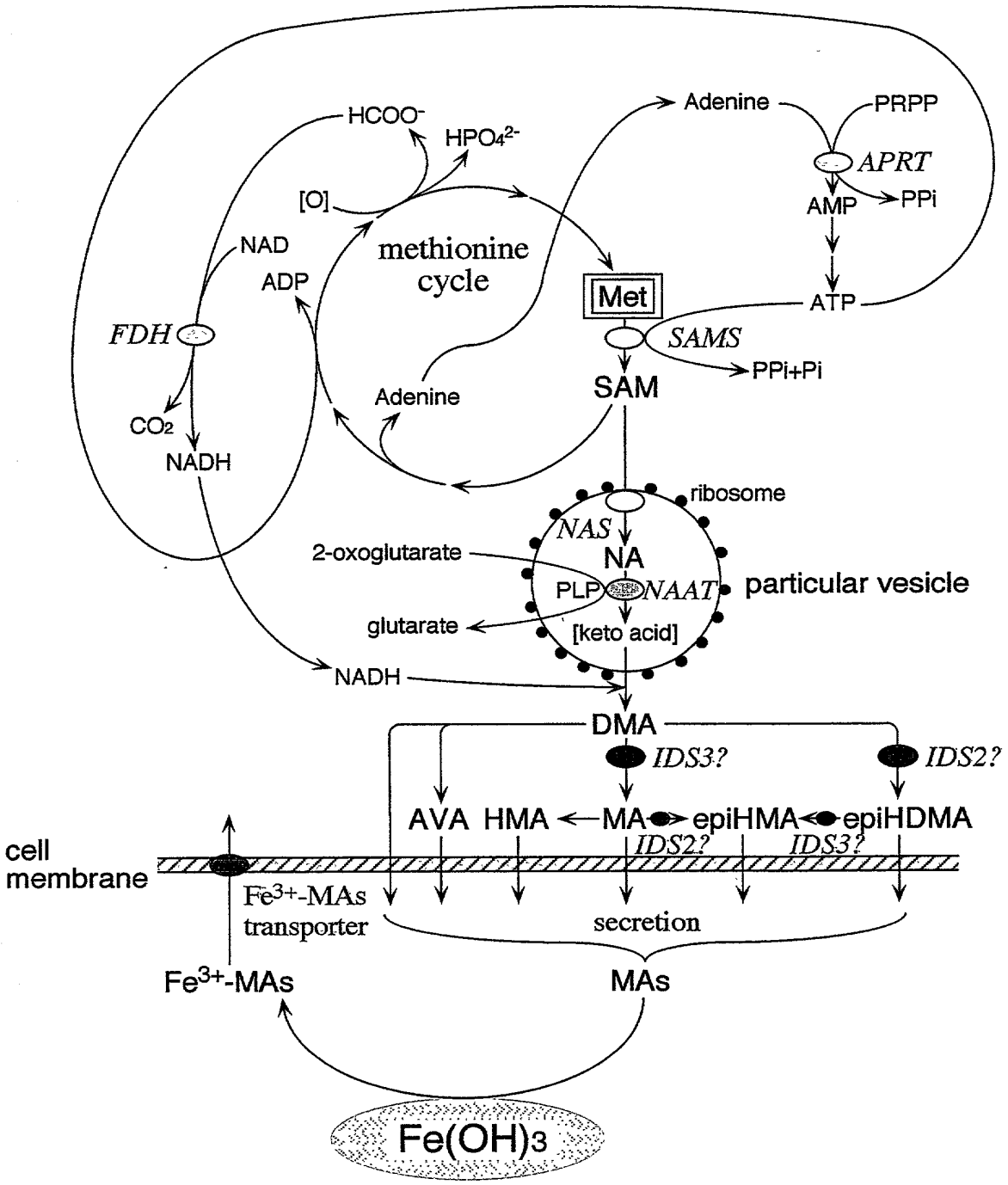
FIG. 1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ADP
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CO2
NADH
methionine cycle
Adenine
PRPP
APRT
AMP
PPi
ATP
Met
SAMS
PPi+Pi
SAM
Adenine
ribosome
NAS
NA
2-oxoglutarate
PLP
NAAT
particular vesicle
glutarate
[keto acid]
NADH
DMA
IDS3?
IDS2?
AVA
HMA
MA
epiHMA
epiHDMA
IDS2?
IDS3?
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secretion
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Fe(OH)3

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FIG. 2

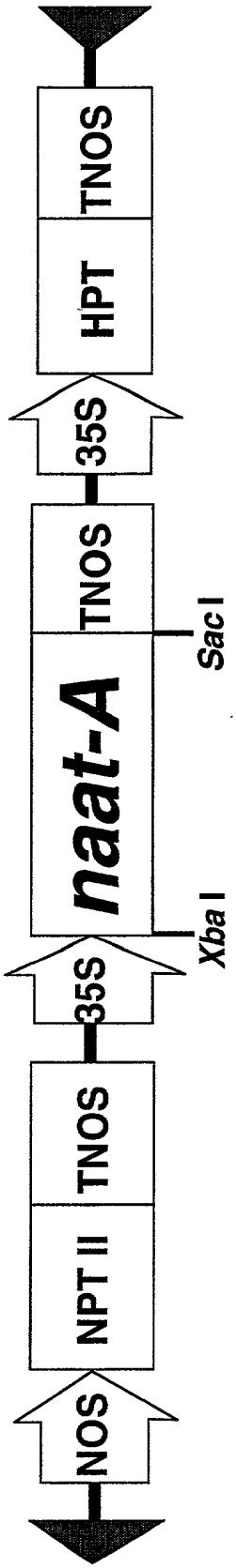

FIG. 3

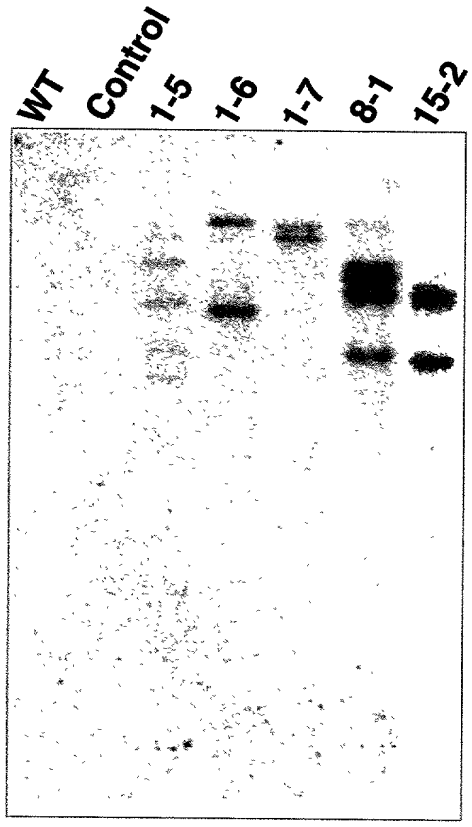

FIG. 4

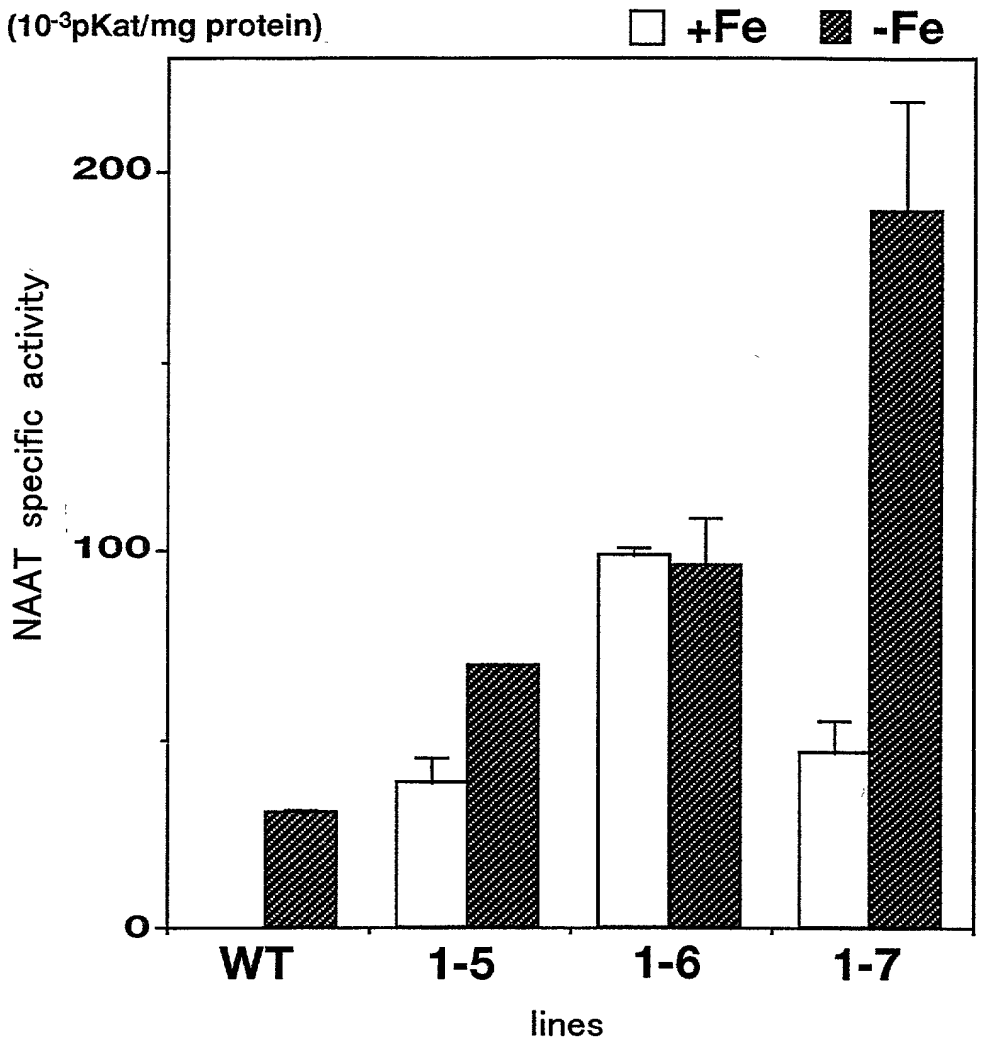
(10-3pKat/mg protein)
+Fe
-Fe
200
100
0
NAAT specific activity
WT
1-5
1-6
1-7
lines

FIG. 5

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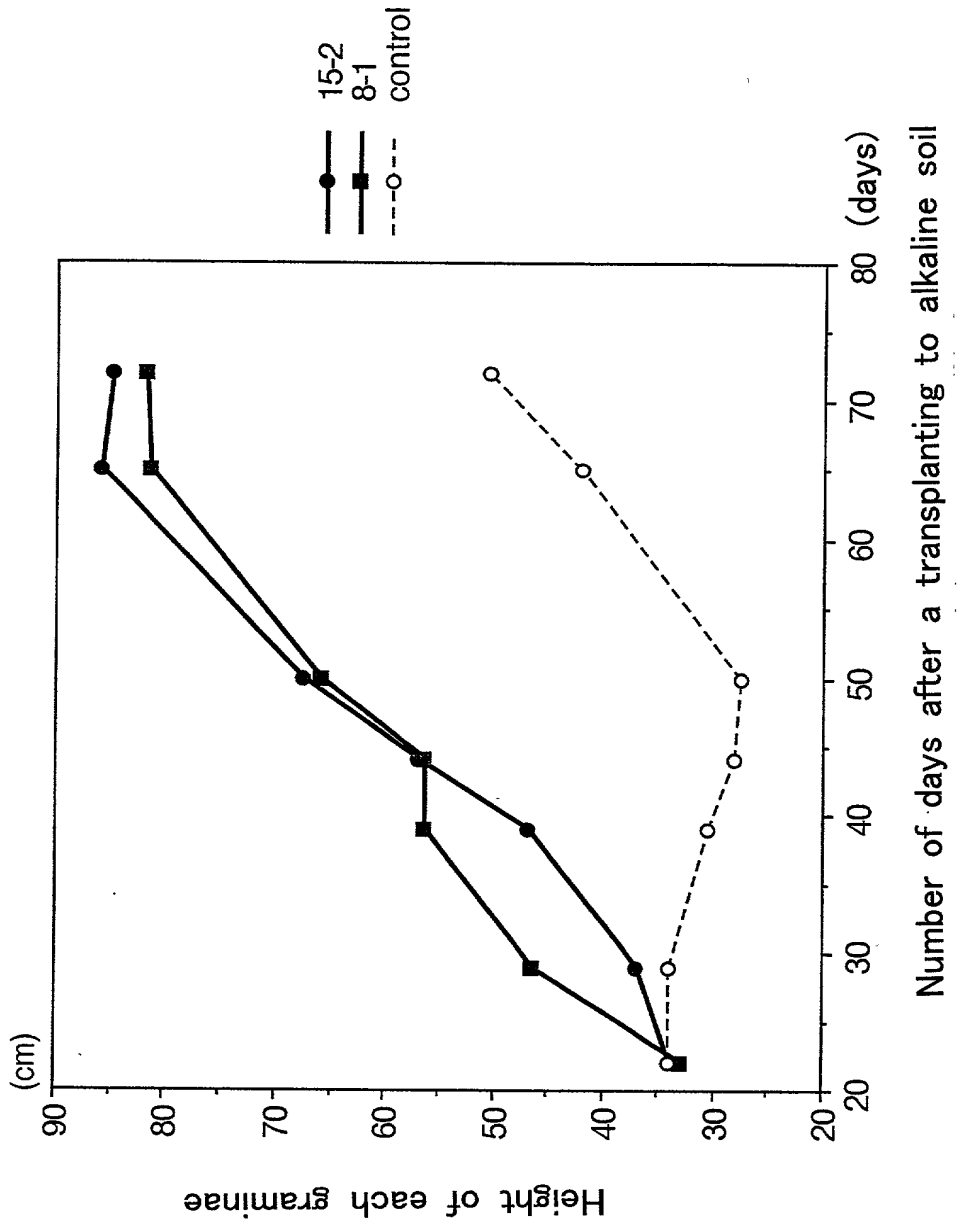
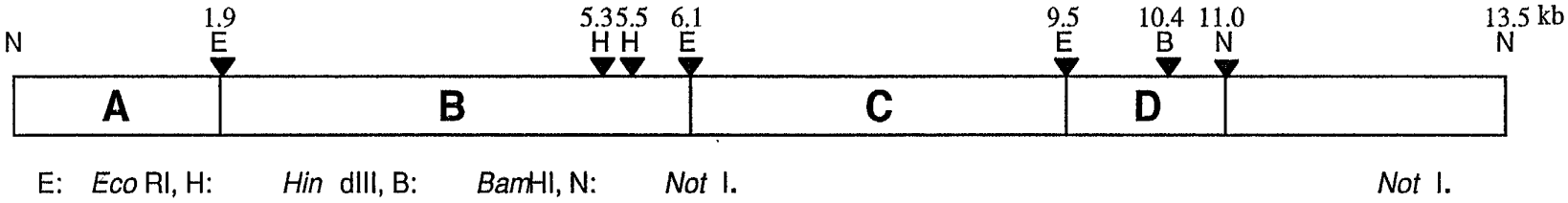

15-2
8-1
control
Height of each graminae
(cm)
90
80
70
60
50
40
30
20
20
30
40
50
60
70
80 (days)
Number of days after a transplanting to alkaline soil

F I G. 6

2092h0" E8Z6T00T

FIG. 7



E: *Eco* RI, H: *Hin* dIII, B: *Bam*HI, N: *Not* I. *Not* I.

The *Not* I site on both sides is the *Not* I located at the arm of λFIX II

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FIG. 8

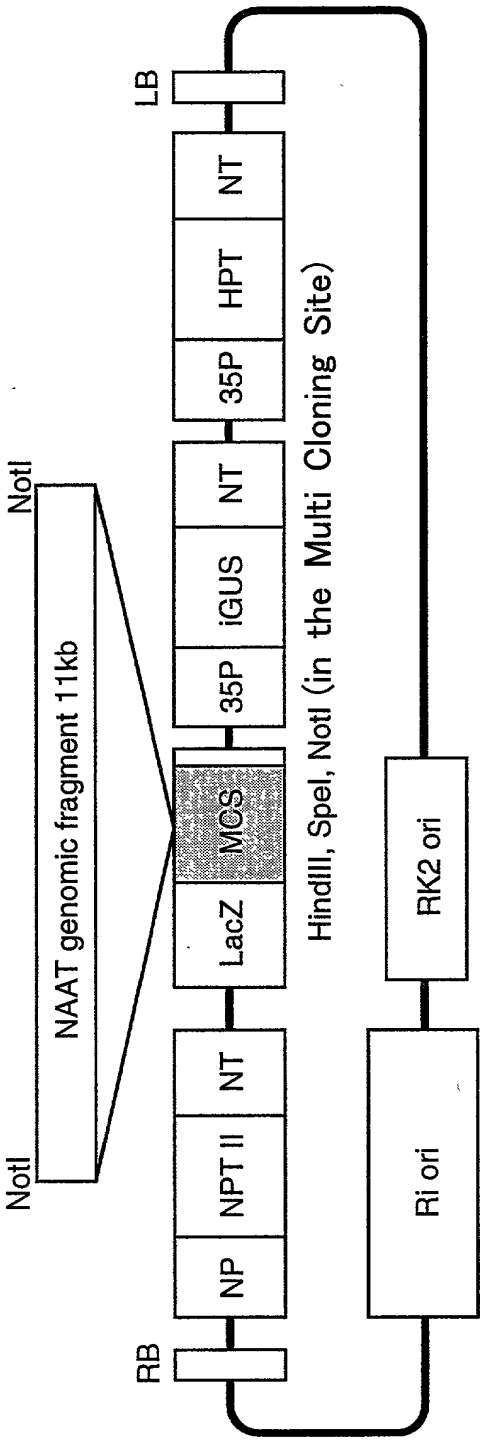

FIG. 9

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FIG. 10

```
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                                                  M  A  T  V  NAAT-B

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AGAGGAGGAGGAGGCGGTGGAGTGGAATTTCGCGGGTGCCAAGGACGGCGTGCTGGCGGC
  E  E  E  E  A  V  E  W  N  F  A  G  A  K  D  G  V  L  A  A

GACGGGGGCGAACATGAGCATCCGGGCGATACGGTACAAGATCAGCGCGAGCGTGCAGGA
  T  G  A  N  M  S  I  R  A  I  R  Y  K  I  S  A  S  V  Q  E

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  K  G  P  R  P  V  L  P  L  A  H  G  D  P  S  V  F  P  A  F

CCGCACGGCCGTCGAGGCCGAGGACGCCGTCGCCGCCGCGCTGCGCACCGGCCAGTTCAA
  R  T  A  V  E  A  E  D  A  V  A  A  A  L  R  T  G  Q  F  N

CTGCTACCCCGCCGGCGTCGGCCTCCCCGCCGCACGAAGgtaacaacaacaacaacacaa
  C  Y  P  A  G  V  G  L  P  A  A  R  S

gaacaatttccttttcgcgtgtcgtgtcgcgcggcaatccatgcatgcgcatgtgccgct
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  L  V  L  G  S  A  P  F  I  P  M  G  V  F  G  H  I  T  P  V

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                        I  S  T  S  I  T  N  Y  L  N  V  S

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                                       V  A  E  V  A  R  K

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F  I  P  M  G  V  F  G  H  I  A  P  V  L  S  I  G  S  L  S

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K  S  W  I  V  P  G  W  R  L  G  W  V  A  V  Y  D  P  T  K

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                                          I  S  T  S  I  T

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                                      V  K  L  N  L  H  L  L

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                                                  G  S  V

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  C  *
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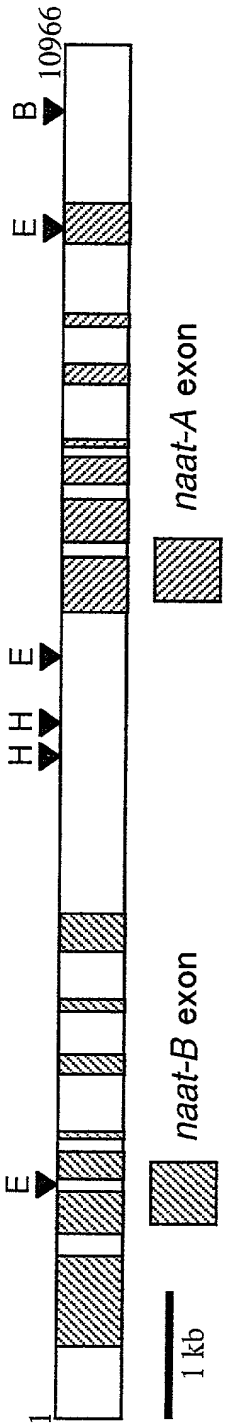
FIG. 11



E: *Eco*RI, H: *Hind*III, B: *Bam*HI.

A schematic view of the obtained genome fragment

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FIG. 12

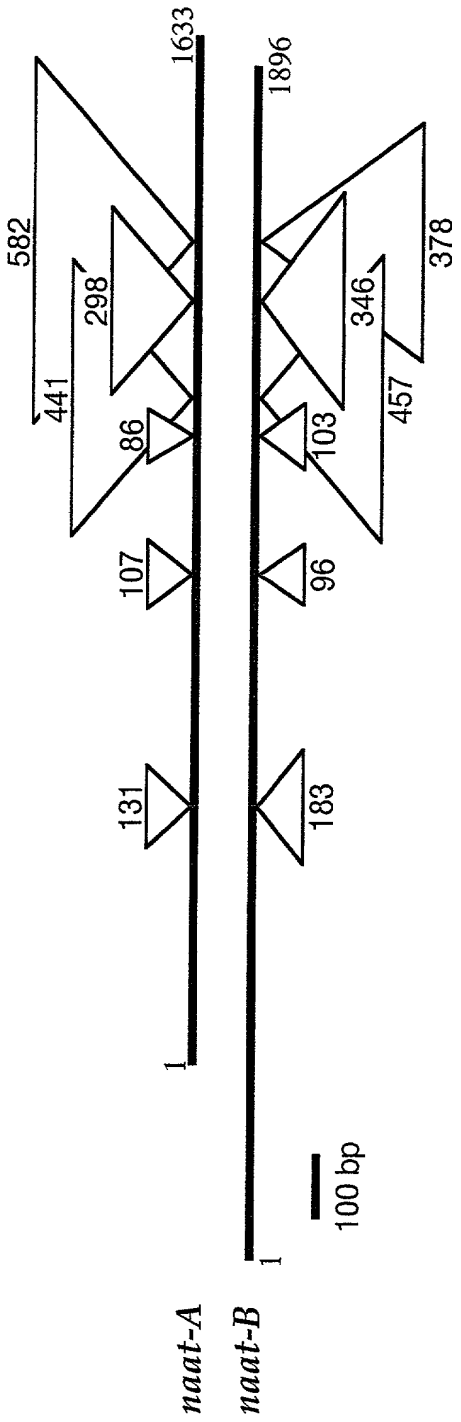

FIG. 13

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FIG. 14

ATVRQSDGVAANGLAVAAAANGKSNGHGVAAAVNGKSNGHGVDADANGKSNGHGVAADAN
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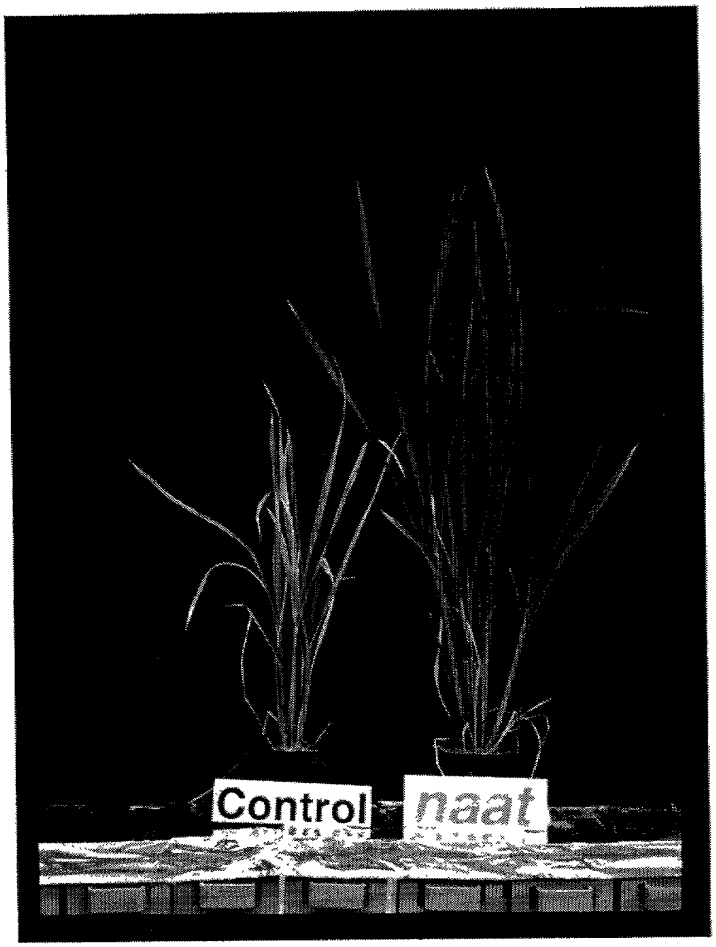
FIG. 15 A

WO 01/36638 PCT/US00/31908

FIG. 15 B



WO 01/05982 PCT/US00/18601

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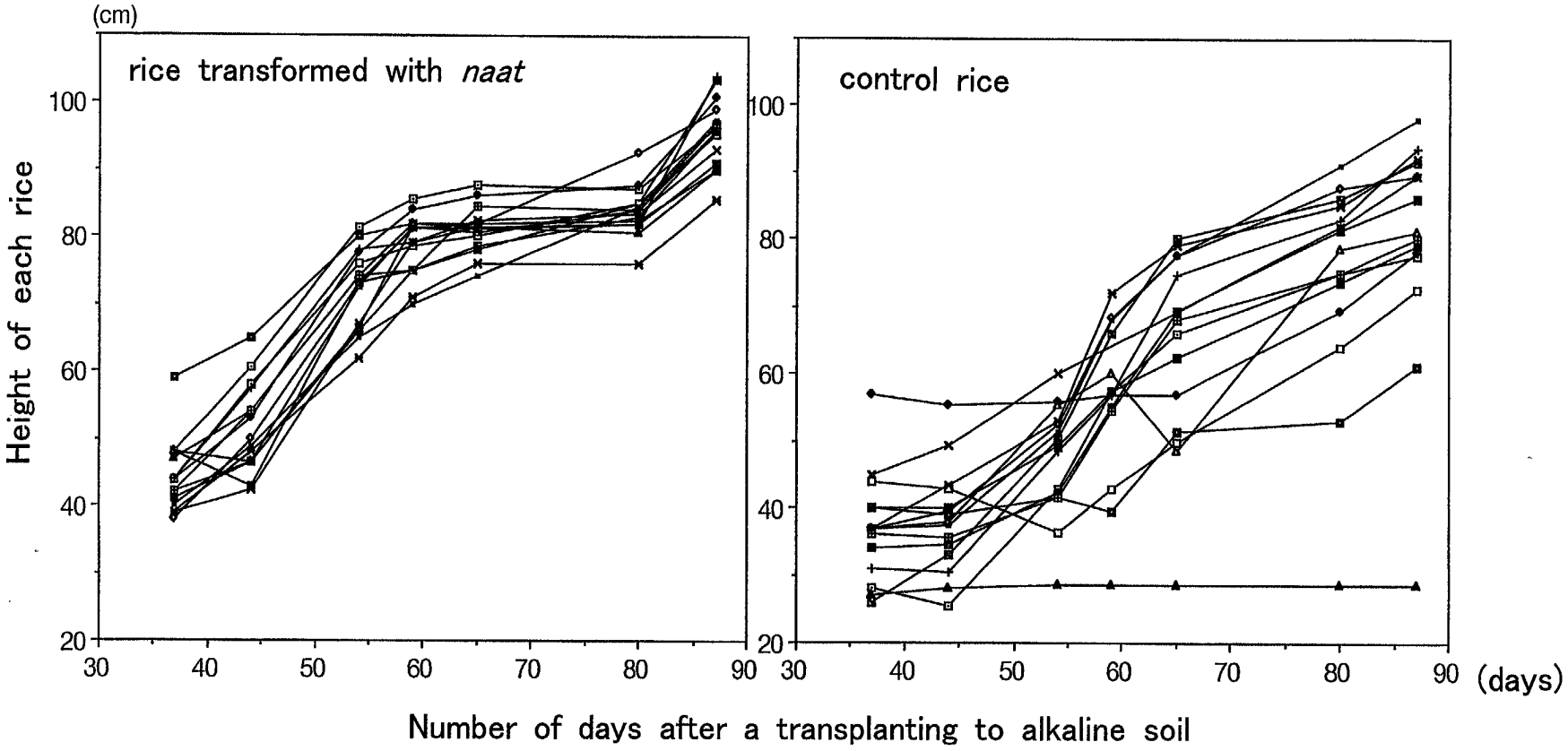
FIG. 16



EP 0 197 783

Rec'd PCT/PTO 26 APR 2002
10/019783

Attorney Docket No. SAE-007

Please type a plus sign in this box: [+]

PTO/SB/01 (3-97)
Approved for use through 6/30/98. OMB 0651-0032
Patent and Trademark Office; US DEPARTMENT OF COMMERCE

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

☒ Declaration submitted with or initial filing

☐ Declaration submitted after initial filing

Attorney Docket No.	***SAE-005***
First Named Inventor	***Satoshi MORI et al.***
COMPLETE IF KNOWN	
Application No.	To be assigned
Filing Date	Concurrently herewith
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (only if one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

THE MANUFACTURING OF IRON DEFICIENT RESISTANT GRASSES

(Title of the Invention)

the specification of which

☐ is attached hereto

or

☒ was filed on ***July 4, 2000***, as United States Application Number or PCT International Application Number: ***PCT/JP00/04425***.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YY)	Priority Not claimed	Certified Copy Attached YES	NO
11-190318	***JAPAN***	***07/05/1999***		☐	☐
				☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number (s)	Filing Date (MM/DD/YY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

10019783 - 042602

PD 908341

Attorney Docket No. SAE-007

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PTO/SB/01 (3-97)
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DECLARATION - Utility Or Design Patent Application

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the matter provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☒ Customer Number 23353
and
☒ Registered practitioner(s) name/registration number listed below

Place Customer Number Bar Code Label Here

Name	Registration No.	Name	Registration No.
Richard D. Grauer	22,388	Kevin D. Rutherford	40,412
David T. Nikaido	22,663	Glenn E. Forbis	40,610
Ronald P. Kananen	24,104	Kristin L. Murphy	41,212
H. Lawrence Smith	24,900	Matthew J. Russo	41,282
Ralph T. Rader	28,772	Robert S. Green	41,800
Carl Schaukowitch	29,211	David K. Benson	42,314
Michael D. Fishman	31,951	Brian K. Dutton	47,255
Joseph V. Coppola, Sr.	33,373	Eugene G. Byrd	47,361
Michael B. Stewart	36,018		
Alexander D. Rabinovich	37,425		

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to ☒ Customer Number or Bar Code Label **23353** or ☐ Correspondence Address below

Name	Rader Fishman & Grauer PLLC				
Address	**1233 20th Street, N.W., Suite 501**				
City, State, Zip	**Washington, D.C. 20036**				
Country	US	**Telephone**	**202-955-3750**	**Fax**	**202-955-3751**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of First Inventor ☐ A petition has been filed for this unsigned inventor.

Given Name (first and middle [if any])	Family Name or Surname
Satoshi	*MORI*

Inventor's Signature	Satoshi Mori					**Dated**	December 27, 2001
Residence: City	*Tokyo*	**State**	JPX	**Country**	*Japan*	**Citizenship**	*Japan*
Post Office Address	*5-32-2-206, Hongo, Bunkyo-ku*						
City	*Tokyo*	**State**		**Zip**	113-0033	**Country**	*Japan*

☒ Additional inventors are being named on the 1 supplemental additional inventor(s) sheet(s) PTO/SB/02A attached hereto.

PD 908341

Attorney Docket No. SAE-007

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PTO/SB/02A (3-97)
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Name of Inventor	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Hiromi			*NAKANISHI*				
Inventor's Signature	Hiromi Nakanishi				**Dated** December 27, 2001		
Residence: City	*Tokyo* JPX	**State**		**Country**	*Japan*	**Citizenship**	*Japan*
Post Office Address	*5-32-20-308, Sendagi, Bunkyo-ku*						
City	*Tokyo*	**State**		**Zip**	113-0022	**Country**	*Japan*

Name of Inventor	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Michiko			*TAKAHASHI*				
Inventor's Signature	Michiko Takahashi				**Dated** December 27, 2001		
Residence: City	*Tokyo* JPX	**State**		**Country**	*Japan*	**Citizenship**	*Japan*
Post Office Address	*3-18-4, Kohinata, Bunkyo-ku*						
City	*Tokyo*	**State**		**Zip**	112-0006	**Country**	*Japan*

Name of Inventor	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Naoko			*NISHIZAWA*				
Inventor's Signature	Naoko Nishizawa				**Dated** December 27, 2001		
Residence: City	*Tokyo* JPX	**State**		**Country**	*Japan*	**Citizenship**	*Japan*
Post Office Address	*1-37-9-705, Hakusan, Bunkyo-ku*						
City	*Tokyo*	**State**		**Zip**	113-0001	**Country**	*Japan*

10/019783
JC13 Rec'd PCT/PTO 0 4 JAN 2002

SEQUENCE LISTING

<110> Japan Science and Technology Corporation
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TAKAHASHI, Michiko
NISHIZAWA, Naoko
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Replacement Sheet

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EP 1 396 543 A2

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